Soluble receptor for advanced glycation end-products attenuates sepsis-induced acute kidney injury

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Soluble receptor for advanced glycation end-products attenuates sepsis-induced acute kidney injury

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Sun Young Park

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ABSTRACT

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Background:

Acute kidney injury (AKI), an important complication of sepsis, is considered to be resulted from immune-mediated tubular damage. Receptor for advanced glycation end products (RAGE) has been implicated in the pathogenesis of numerous inflammatory conditions including sepsis. Meanwhile, soluble RAGE (sRAGE) competitively inhibits the binding of RAGE ligands to RAGE, and thus attenuates the development of RAGE-induced inflammatory cascades. However, little is known about the efficacy of sRAGE in septic AKI (S-AKI). In this study, I investigated the possible therapeutic role of sRAGE in S-AKI models, both *in vivo* and *in vitro*.

Methods:

In vivo, C57/BL6 mice were subjected to cecal ligation and puncture (CLP) or sham operation (control), and sacrificed after 24 hr. CLP mice were injected either with diluent or sRAGE intraperitoneally (CLP+sRAGE) 1 hr before operation. *In vitro*, in addition, NRK-52E cells were incubated with DMEM media with or without lipopolysaccharide (LPS, 1 μg/ml). To examine the effect of RAGE inhibition on LPS-induced tubular cell injury, NRK-52E cells were also treated with sRAGE (1 μg/ml) or RAGE siRNA. Blood urea nitrogen (BUN) and serum creatinine (Cr) levels were determined to evaluate renal function. RAGE-associated signaling molecule and apoptosis-related protein expressions were evaluated by Western blot analysis and immunohistochemistry.

Results:

BUN and serum Cr levels were significantly higher in CLP model compared to control mice, and these increments were significantly abrogated in CLP+sRAGE mice ($P < 0.05$). Renal MyD88, phospho-ERK, phospho-p-38, and phospho-JNK protein expression in the CLP group were also significantly increased compared to the control group, and these changes were significantly ameliorated by sRAGE treatment in CLP mice. Compared to the control group, moreover, apoptosis-related protein expressions were significantly increased in the kidney of CLP mice, and sRAGE treatment significantly attenuated these increases in the CLP mice kidney. *In vitro*, HMGB1 and RAGE protein expression were significantly increased in LPS-stimulated NRK-52E cells, and sRAGE significantly abrogated these LPS-induced of HMGB1 and RAGE protein expression. Similarly, RAGE-associated activation of mitogen-activated

protein kinase and increase in apoptosis-related protein expression in LPS-stimulated cells were significantly ameliorated by sRAGE. Furthermore, the increase in nuclear translocation of NF-κB and ICAM-1 protein expression by LPS were significantly attenuated by sRAGE in NRK-52E cells.

Conclusions:

These findings suggest that RAGE plays an important role in S-AKI and its inhibition by sRAGE may be a potential therapeutic target for AKI in severe sepsis.

Key words: receptor for advanced glycation end products (RAGE), soluble RAGE (sRAGE), sepsis, acute kidney injury (AKI)

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I. INTRODUCTION

Acute kidney injury (AKI) is a common and serious problem in critically ill patients. In addition, even though AKI is known to be an independent risk factor for mortality, its incidence rates are gradually increasing in nowadays.[1, 2] Among various etiologies of AKI, sepsis or septic shock is the most frequent causes of AKI especially in critically ill patients in an intensive care unit, which is composed of approximately 35% of AKI.[3, 4] Moreover, the mortality rates of septic AKI (S-AKI) patients are extremely higher compared to AKI patients resulted from other etiologies despite recent advanced therapy.[2-5] Therefore, an optimal treatment for S-AKI is still a matter to be established.

To date, the pathophysiology of S-AKI remains still elusive. Previous studies showed that renal tubular cells after ischemic injury associated with sepsis were

detached from the tubular basement membrane and then form tubular casts, resulting in acute tubular necrosis in S-AKI.[6, 7] Recently, however, it has become increasingly recognized that many pro- and anti-inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-10, rather than ischemic insult in renal tubules, play a more important role in the development of sepsis-induced tubular injury, suggesting that inflammation is a key event contributing to the occurrence of AKI in patients with sepsis.[8-12]

Meanwhile, it has also been demonstrated that pattern recognition receptors, such as the toll-like receptors (TLRs) or receptor for advanced glycation end-products (AGEs) (RAGE), are implicated in the development of inflammation in the kidney.[13-16] RAGE, a signal transducing-receptor binding to AGEs, is a member of the immunoglobulin (Ig) superfamily, which consists of one Ig like V-type domain and two Ig like C-type domain in the extracellular portion, a single transmembrane-spanning domain, and cytoplasmic tail.[14, 17] Binding of RAGE to not only AGEs but also high-mobility group B1 (HMGB1), S100/calgranulin, and β2-integrin Mac-1, activates signaling transduction pathways and transcription factors, including mitogen-activated protein (MAP) kinase, as well as the downstream activation of nuclear factor-kappa B (NF-κB), which then leads to the production of reactive oxygen species (ROS) and pro-inflammatory cytokines, and consecutively induces inflammation.[14-20]

Soluble RAGE (sRAGE), which is RAGE without membrane anchor and cytoplasmic portion and a circulating form of RAGE, is produced endogenously by ectodomain shedding of RAGE or alternative splicing of RAGE mRNA transcript.[14] It acts as a decoy receptor by competitively binding to RAGE, and thus plays an antagonistic role to RAGE. Recently, blocking of RAGE signaling

using sRAGE has been revealed to be a potential therapeutic candidate for various diseases associated with inflammation such as left ventricular hypertrophy, atherosclerosis, Alzheimer's disease, and arthritis.[21, 22]

HMGB1, as one of endogenous ligands of RAGE, is found to be involved in the activation of RAGE signaling in sepsis.[23] In animal models of sepsis induced by lipopolysaccharide (LPS), increased serum HMGB1 concentrations were closely related to the severity of sepsis and administration of a blocking antibody to HMGB1 improved the survival rates of these anmials.[24] LPS is well-known exogenous pathogen induced by endotoxin in sepsis and also serves as a ligand of TLRs, which also mediates sepsis-induced immune responses.[25] A recent study by Yamamoto et al. showed that LPS activated RAGE as well as TLRs, suggesting that inhibiting RAGE signaling might abrogate the increase in HMGB1 levels and LPS-induced inflammatory response in sepsis.[13] In the present study, I aimed to investigate the therapeutic effects of sRAGE on LPS-induced renal tubular cells *in vitro* and in experimental S-AKI animals *in vivo*.

II. MATERIALS AND METHODS

1. Measurement of serum sRAGE levels in human subjects

Blood samples from S-AKI patients initiating continuous renal replacement therapy (CRRT) were collected after obtaining the approval from the Institutional Review Board (IRB) of Yonsei University Heath System Clinical Trial Center (IRB No; 10-0440), all participants provided written informed consent. Blood were also collected from age- and gender- matched healthy controls and patients with end-stage renal disease (ESRD). sRAGE (Human RAGE immunoassay; R&D Systems, Inc., Minneapolis, MN, USA) and extracellular newly identified RAGE-binding protein (EN-RAGE, CircuLexTM S100A12/EN-RAGE ELISA kit, CycLex Co., Ltd., Nagano, Japan) were measured using commercially available ELISA kit following the manufacturer's protocols.

2. Animal study

All animal procedures were conducted under protocols approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, Seoul, Korea. Thirty-two C57/BL6 mice of 9-10 weeks old were used in this study. To generate sepsis animals, cecal ligation and puncture (CLP) were performed as previously reported.[26] Mice were anesthetized with ketamine and xylazine, midline laparotomy, and cecal ligation were performed at 1 cm from the cecum tip using 4.0 silk, and the cecum was punctured twice by 21-G needle. Eight mice from the sham operation and CLP groups were injected intraperitoneally with either diluent or 1 μg/kg of sRAGE 1 hr before operation.

Purified sRAGE protein was purchased from A&R Therapeutics (Daejeon, Korea), and purification protocol was previously described.[27] Mice were sacrificed at 24 hr after sham or CLP surgery, and blood was collected and the kidney was removed for histological evaluation and molecular biological analysis. Blood urea nitrogen (BUN) and serum creatinine concentrations were determined by a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan). Interleukin-6 (IL-6) levels were measured by mouse IL-6 ELISA kit (R&D systems, Minneapolis, MN, USA) using the manufacturer's protocol.

3. Cell culture study

NRK-52E cells, immortalized rat tubular epithelial cells, were maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 26 mM NaHCO3, at 37ºC in humidified 5% CO_2 in air. Subconfluent NRK-52E cells were serum restricted for 24 hr, after which the media were replaced by serum-free medium containing 1 μg/ml lipopolysaccharide (Sigma Chemical Co., St. Louis, MO, USA) with or without 1 μg/ml sRAGE. RAGE siRNA (100 nM) was also used in the current study. It was transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. At 24 hr after the media change, the cells were harvested. To investigate nuclear translocation of NF-κB, nuclear and cytosolic fractions were separated from cultured cells harvested from plates using commercially available kit (Thermoscientific, Waltham, MA, USA).

4. Western blot analysis

For western blot, 30 μg of protein extracted from the homogenized whole kidney and cultured cells were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in an 8%-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA,USA), and the membrane was then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) at room temperature for 1 hr, followed by an overnight incubation at 4°C in a 1:1000 dilution of primary antibodies to HMGB1 (Cell Signaling, Inc., Beverly, MA, USA), RAGE (Abcam, Cambridge, UK), Bax, Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), cleaved caspase-3, cleaved PARP, MyD88, phospho-ERK/ERK, phosphor-p38/p38 MAPK, phospho-JNK/JNK, phospho-NF-κB/NF-κB (Cell Signaling, Inc., Beverly, MA, USA), ICAM-1 (R&D systems, Minneapolis, MN, USA), or β-actin (Sigma Chemical Co.). The membrane was then washed once for 15 min and twice for 5 min in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked donkey anti-goat IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

5. Histological examination

Slices of the kidney were fixed in 10% neutral-buffered formalin, processed in the standard manner, and 5 μm-thick sections of paraffin-embedded tissues were utilized for periodic acid-schiff's (PAS) and immunohistochemical (IHC) staining. PAS staining was used for the analysis of tubular injury score by a standard method as previously reported.[28] A semi-quantitative scores for tubular injury, including tubular epithelial cell swelling, loss of brush border, and necrotic tubules, were determined on a scale of 0-4+: 0, normal;, 1+, <25% of the tubules;, 2+, 25-50% of the tubules;, 3+, 50-75% of the tubules;, and 4+, more than 75% of the tubules. The sections of the kidneys were examined at least 20 tubulointerstitial fields under x 200 magnification per specimen by two investigators in a blinded manner. For IHC staining of HMGB1 and RAGE, slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Primary antibodies for HMGB1 and RAGE were diluted to the appropriate concentrations with 2% casein in bovine serum albumin and then add to the slides, followed by an overnight incubation at 4°C. After washing, a secondary antibody was added for 20 min, and the slides were washed and incubated with a tertiary rabbit-PAP complex (Dako Denmark A/S, Glostrup, Denmark) for 20 min. Deaminobenzidine was added for 2 min and the slides were counterstained with hematoxylin. A semi-quantitative score of staining intensity was determined by examining at least 20 tubulointerstitial fields under x 400 magnification by two investigators in a blinded fashion, using a digital image analyzer (MetaMorph version 4.6r5, Universal Imaging, Downingtown, PA). The staining score was obtained by

multiplying the intensity of staining by the percentage of tubulointerstitium staining for that intensity; these numbers were then added for each experimental animal to give the staining score [= Σ (intensity of staining) x (% of tubulointerstitium with that intensity)].

IHC staining for macrophages was done using an antibody for F4/80 (Abcam, Cambridge, UK). The quantification of F4/80-positive cells was expressed as a number of cells per high power field (HPF), which was counted in at least 20 randomly chosen sections under x 400 magnification by two investigators in a blinded fashion.[29]

6. TUNEL assay and Hoechst 33342 staining

In addition to the changes in the protein expression of apoptosis-related molecules, apoptosis was also identified within the kidney by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a commercially available kit (Chemicon International, Billerica, MA, USA) and in cultured NRK-52E cells seeded on cover slips by Hoechst 33342 (Molecular Probes, Eugene, OR) staining. Apoptosis was defined as TUNEL-positive cells within the tubules and the presence of nuclear condensation on Hoechst staining. The percentage of TUNEL-positive tubular cells in formalin-fixed renal tissue and NRK-52E cells with nuclear condensation were determined by examining at least 30 randomly chosen fields of the kidney sections and 300 cells /conditions, respectively, at x 400 magnification.

7. Fluorescence-activated cell sorting (FACS) analysis

$CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells were counted by FACS as

previously described.[30] Briefly, immune cells from splenocytes in animal were collected in RPMI medium (Invitrogen). T cells were isolated using a CD4 T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, NRW, Germany). Treg cells were stained with anti-CD4−FITC and anti-CD25−PerCP-Cy5.5 (BD Biosciences, San Diego, CA, USA), and the cells were fixed and permeabilized with FoxP3 staining buffer (eBioscience, San Diego, CA, USA), followed by staining for FoxP3−PE (eBioscience). Flow cytometric analysis of surface staining was conducted with an FACSVERSE (BD Biosciences), and the results were analyzed using FACSuite software (BD Biosciences).

8. Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 20.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the ANOVA and Kruskal-Wallis non-parametric test for multiple comparisons. Significant differences by the ANOVA and Kruskal-Wallis test were further confirmed by the Student's t-test and Mann-Whitney U test, respectively. *P*-values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. sRAGE levels are associated with survival in patients with septic AKI

Serum sRAGE concentrations were significantly higher in S-AKI patients (2597.9 ± 3238.9 pg/ml) compared to 30 heathy control subjects (667.3 ± 334.2 pg/ml) and 58 ESRD patients (1507.8 ± 1081.6 pg/ml) ($P < 0.05$), whereas, there was a significant difference in serum levels of EN-RAGE only between S-AKI patients and healthy control subjects (835.6 ± 502.7 vs. 193.9 ± 119.9 ng/ml, $P < 0.01$) (Fig. 1). In addition, serum sRAGE but not EN-RAGE values were significantly higher in survivors relative to non-survivors in S-AKI patients (Table 1).

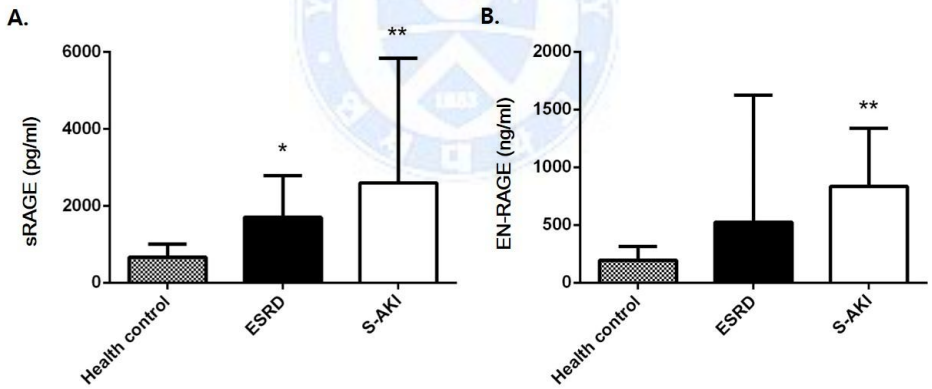

Figure 1. Serum concentrations of sRAGE and EN-RAGE in control, ESRD and S-AKI patients. (A) Serum levels of sRAGE were significantly higher in S-AKI patients compared to healthy control subjects and ESRD patients. (B) Serum EN-RAGE values was significantly higher in S-AKI patients relative to only healthy control subjects. *$P <$ 0.05 vs. healthy control, **$P < 0.01$ vs. healthy control.

Table 1. Baseline characteristics of survivors and non-survivors in septic AKI patients

	Survivors	Non-survivors	*P*
Number	18	42	
Age (years)	58.9 ± 16.9	62.2 ± 12.3	0.39
Male, *n* (%)	9 (50.0%)	29 (69.0%)	0.24
sRAGE (pg/ml)	4558.2 ± 1052.9	1757.8 ± 3324.1	0.02
EN-RAGE (ng/ml)	870.4 ± 131.3	820.6 ± 74.7	0.73

Note: Data are expressed as mean ± SD, or number of patients (percent). Abbreviations: sRAGE, soluble receptor for advanced glycation end-products; EN-RAGE, newly identified RAGE-binding protein.

2. Effect of sRAGE on renal HMGB1 and RAGE protein expression in CLP-induced septic mice

The protein expression of renal HMGB1 and RAGE, assessed by western blot, were significantly increased in the CLP group compared to the control group ($P < 0.05$), and sRAGE pretreatment significantly ameliorated these increases in the kidney of CLP mice ($P < 0.05$) (Fig. 2A). Moreover, IHC staining for HMGB1 and RAGE confirmed the western blot findings. The staining intensities for HMGB1 and RAGE within the tubulointerstitium were significantly higher in the CLP group compared to control and control+sRAGE mice, and these changes in CLP mice were significantly attenuated by the administration of sRAGE (Fig. 2B).

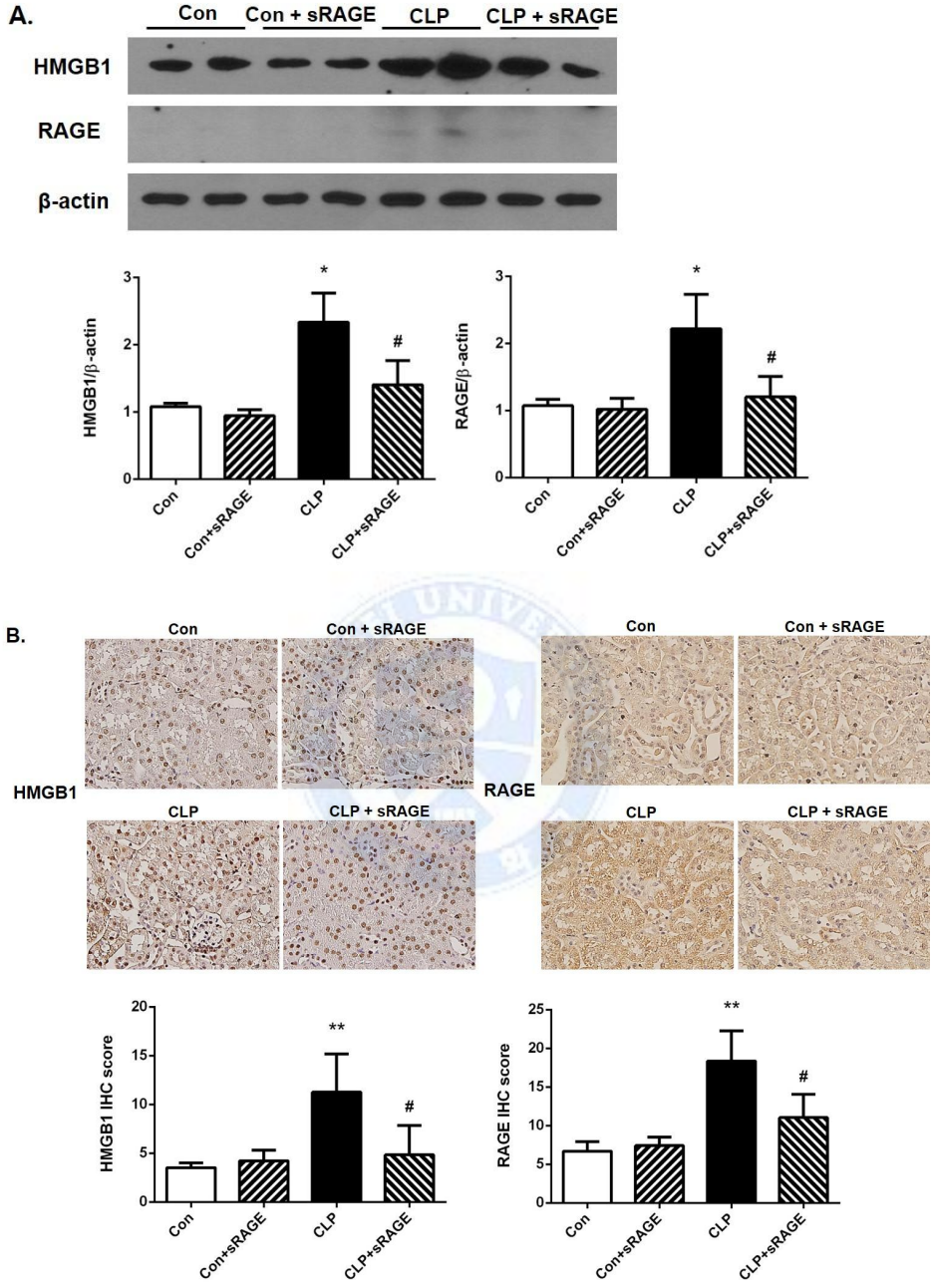

Figure 2. Renal HMGB1 and RAGE protein expression in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice. (A) A representative western blot of

HMGB1 and RAGE in the Con, Con+sRAGE, CLP, and CLP+sRAGE groups (Representative of five blots). The protein expression of renal HMGB1 and RAGE were significantly increased in the CLP group compared to the Con group, and sRAGE pretreatment significantly ameliorated these increases in the kidney of CLP mice. In contrast, there were no significant differences in HBMG1 and RAGE protein expression between the Con and Con+sRAGE groups. (B) Immunohistochemical staining for HMGB1 and RAGE revealed a similar pattern to the results of western blot. *$P < 0.05$ and **$P < 0.01$ vs. Con group, $^{\#}P < 0.05$ vs. CLP group.

3. sRAGE improves renal function in CLP-induced septic mice

At the time of sacrifice, BUN, and serum creatinine, and IL-6 concentrations were significantly higher in the CLP group (62.4±24.2, 0.44±0.21 mg/dl, and 109.7±114.2 pg/ml, respectively) compared to the control group (10.2±4.2, 0.10±0.02 mg/dl, and 5.7±10.3 pg/ml, respectively) ($P < 0.05$), and sRAGE pretreatment significantly abrogated the increases in BUN, creatinine, and IL-6 levels in CLP mice (17.9±5.7, 0.21±0.11mg/dl, and 36.5±28.4 pg/ml, respectively) ($P < 0.05$) (Table 2).

Table 2. BUN, and serum creatinine and IL-6 concentrations in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice

	Con	Con+sRAGE	CLP	CLP+sRAGE
BUN (mg/dl)	10.2±4.2	9.3±6.1	62.4±24.2*	17.9±5.7$^{\#}$
Cr (mg/dl)	0.10±0.02	0.09±0.03	0.44±0.21*	0.21±0.11$^{\#}$
IL-6 (pg/ml)	5.7±10.3	32.4±36.7	109.7±114.2*	36.5±28.4$^{\#}$

Note: Data are expressed as mean ± SD.

*$P < 0.05$ vs. Con group, #$P < 0.05$ vs. CLP group

4. sRAGE abrogates CLP-induced activation of MyD88 and MAP kinase in the kidney

Since RAGE is known to activate a number of intracellular signal transduction pathways, including MyD88 and MAP kinase, I evaluated the impact of sRAGE on these pathways within the kidney in CLP-induced septic mice. Renal MyD88 protein expression was significantly increased in the CLP group compared to the control group. The protein expression of phospho-ERK, phospho-p38, and phospho-JNK were also significantly increased in the kidney of CLP mice relative to control mice. The increases in renal MyD88 protein expression and MAP kinase activation in the CLP groups were significantly ameliorated by the administration of sRAGE (Fig. 3).

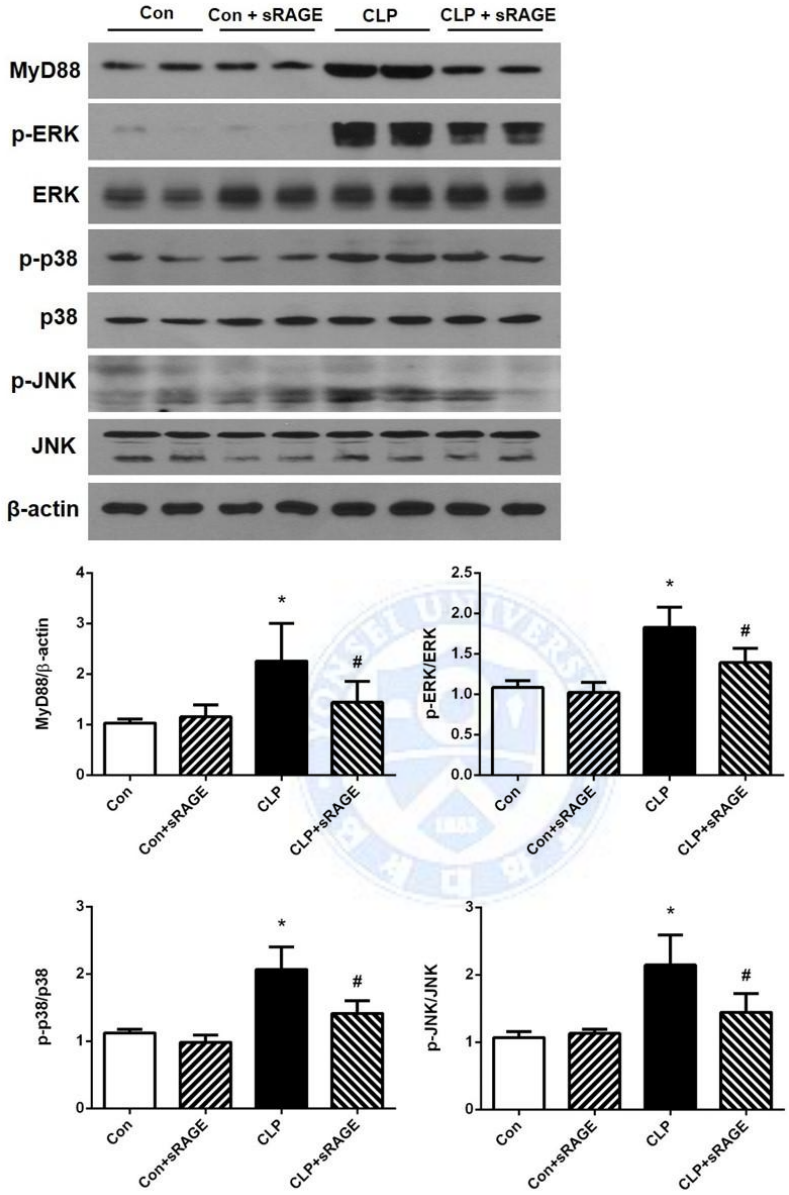

Figure 3. A representative western blot of MyD88, p-ERK/ERK, p-p38/p38 and p-JNK/JNK in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice (Representative of five blots). Renal Myd88, p-ERK, p-p38 and p-JNK protein expression were significantly increased in the CLP group compared to the Con group,

and these changes in the kidney of CLP mice were significantly ameliorated by the administration of sRAGE. *$P < 0.05$ vs. Con group, #$P < 0.05$ vs. CLP group.

5. sRAGE attenuates CLP-induced tubulointerstitial inflammation via inhibiting NF-κB activation

Since RAGE activates NF-κB, which in turn induces and amplifies the inflammation in sepsis, I explored the protein expression of NF-κB and one of its downstream effectors, ICAM-1 in the kidney. NF-κB phosphorylation and ICAM-1 protein expression were significantly increased in the kidney of CLP mice compared to control mice, and sRAGE treatment significantly abrogated the increases in renal phospho-NF-κB and ICAM-1 protein expressions in the CLP group (Fig. 4).

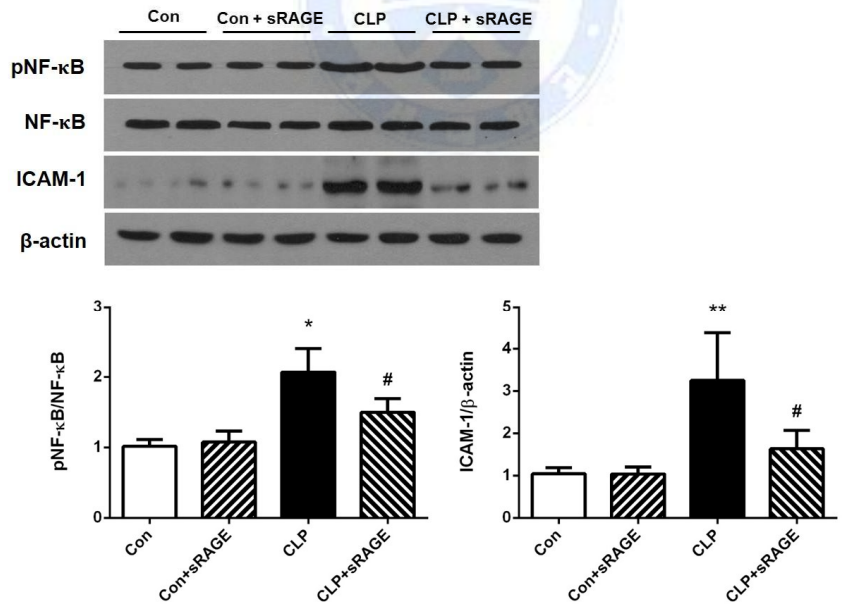

Figure 4. A representative western blot of phospho-NF-κB(p-NF-κB), NF-κB, and

ICAM-1 in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice (Representative of five blots). NF-κB phosphorylation and ICAM-1 protein expression were significantly increased in the kidney of CLP mice compared to control mice, and sRAGE treatment significantly abrogated the increase in renal phospho-NF-κB and ICAM-1 protein expression in the CLP group. *$P < 0.05$ and ** $P < 0.01$ vs. Con group, $^{\#}P < 0.05$ vs. CLP group.

6. sRAGE ameliorates CLP-induced renal tubular cell apoptosis and tubulointerstitial injury

Renal cleaved caspase-3 and cleaved PARP protein expression were significantly increased in the CLP group compared to the control group, and these increases in the kidney of CLP mice were significantly attenuated by the administration of sRAGE (Fig. 5).

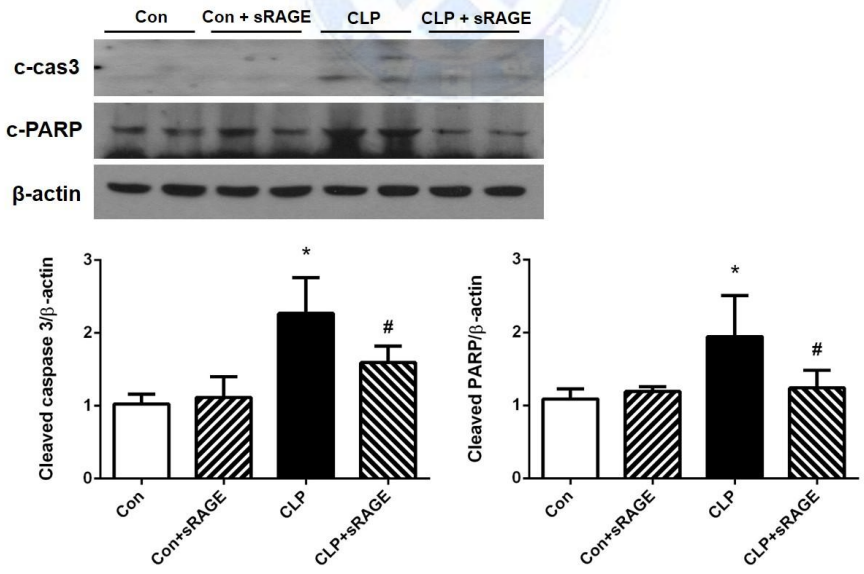

Figure 5. A representative western blot of cleaved caspase-3 (c-cas3) and cleaved

PARP (c-PARP) protein expression in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice (Representative of five blots). Renal c-cas3 and c-PARP protein expression were significantly increased in the CLP group compared to the control group, and these increases in the kidney of CLP mice were significantly attenuated by the administration of sRAGE. $^{*}P < 0.05$ vs. Con group, $^{\#}P < 0.05$ vs. CLP group.

Renal tubular cell apoptosis, assessed by TUNEL assay, was also significantly increased in the CLP group relative to the control group, and sRAGE treatment significantly inhibited CLP-induced renal tubular cell apoptosis. PAS staining demonstrated that tubulointerstitial injury was also significantly abrogated in sRAGE-treated CLP mice. Furthermore, the anti-inflammatory effect of sRAGE on CLP-induced renal tubulointerstitial injury was examined by IHC staining for macrophage using F4/80 antibody. The number of infiltrated F4/80-positive cells was significantly higher in the CLP group compared to the control group, and sRAGE treatment significantly ameliorated the number of F4/80-positive cells (Fig. 6). Taken together, it was surmised that sRAGE exerted a renoprotective effect partly via protecting renal tubular cell from apoptosis in S-AKI.

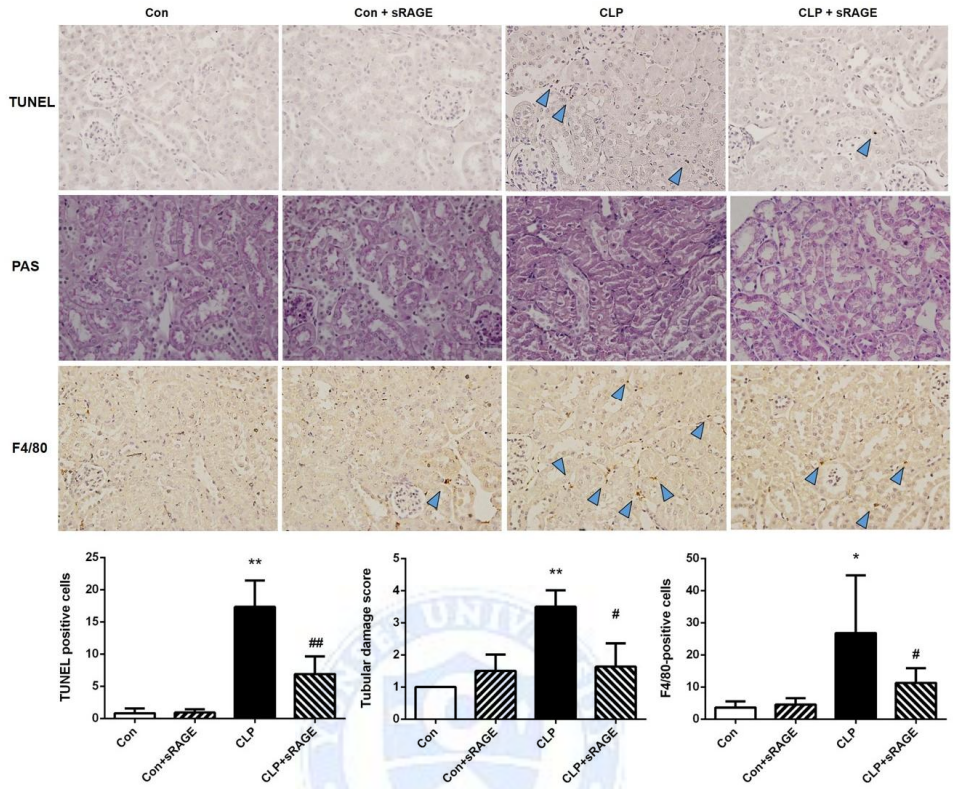

Figure 6. Apoptosis assessed by terminal deoxynucleotidyl transferase-medicated dUTP nick end labeling (TUNEL) assay and IHC staining for F4/80 in control (Con), Con+sRAGE, CLP, and CLP+sRAGE mice. The number of apoptotic cell (arrowheads) was significantly higher in the CLP group compared to the control group, and sRAGE treatment significantly inhibited CLP-induced renal tubular cell apoptosis. PAS staining confirmed that tubulointerstitial injury was also significantly abrogated in sRAGE-treated CLP mice. The number of infiltrated F4/80-positive cells was significantly higher in the CLP group, and sRAGE treatment significantly ameliorated F4/80-positive cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Con group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CLP group.

7. sRAGE augments the increase in $CD4^+CD25^+Foxp3^+$ Treg cells in splenocytes of CLP mice

FACS analysis with splenocytes found that the number of $CD4^+CD25^+Foxp3^+$ Treg cells was increased in the CLP group compared to the control group, and this increase was further augmented by sRAGE pretreatment in CLP mice (Fig. 7).

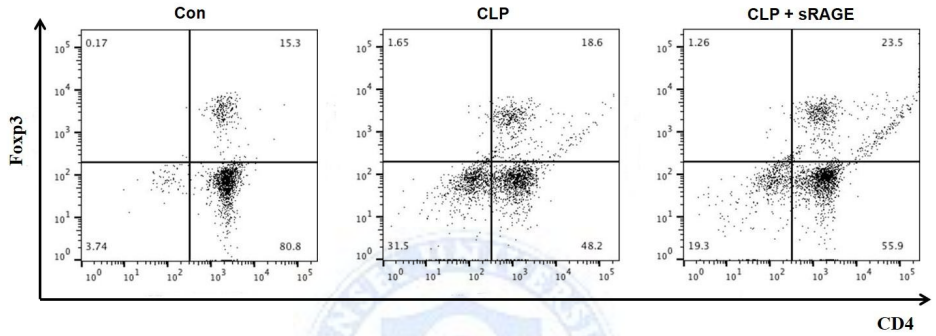

Figure 7. FACS analysis for $CD4^+CD25^+Foxp3^+$ Treg cells in splenocyte. The number of $CD4^+CD25^+Foxp3^+$ Treg cells was increased in the CLP group compared to the control group (Con), and this increase was further augmented by sRAGE pretreatment in CLP mice.

8. LPS induces HMGB1 and RAGE protein expression in NRK-52E cells

The protein expression of HMGB1 and RAGE were significantly increased in LPS-stimulated NRK-52E cells, and these increases in HMGB1 and RAGE protein expression were significantly attenuated by the administration of sRAGE in a dose-dependent manner (Fig. 8).

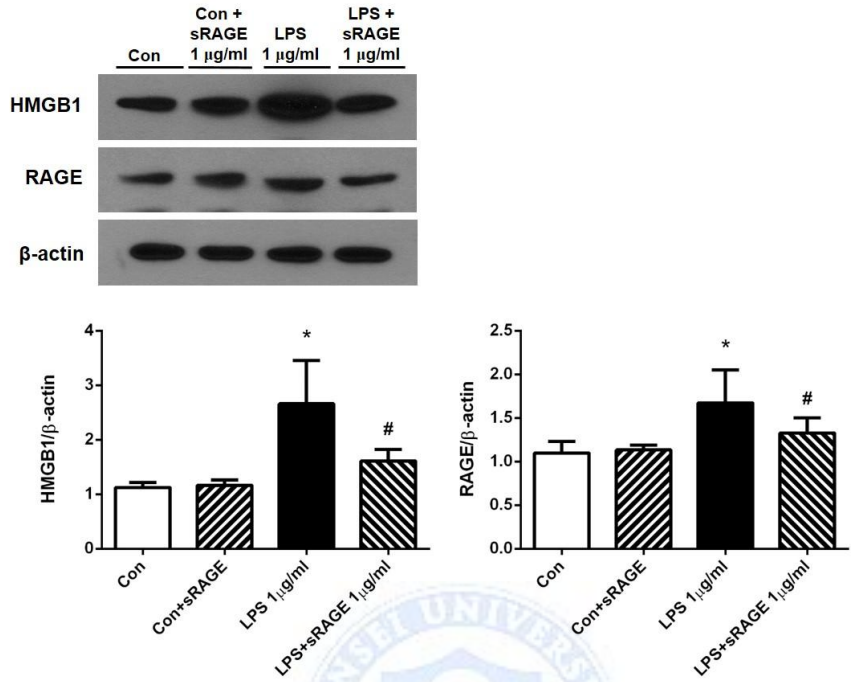

Figure 8. A representative western blot of HMGB1 and RAGE protein expression in control (Con), Con+sRAGE (1 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), and LPS+sRAGE groups. The protein expression of HMGB1 and RAGE were significantly increased in LPS-stimulated NRK-52E cells compared to control cells, and these increases in HMGB1 and RAGE protein expression were significantly attenuated by the administration of sRAGE in a dose-dependent manner. $^{*}P < 0.05$ vs. Con group, $^{\#}P < 0.05$ vs. LPS group.

9. sRAGE attenuates LPS-induced RAGE signaling in NRK-52E cells

Compared to control cells, MyD88, phospho-ERK, phsopho-p38, and phospho-JNK protein expression were significantly increased in NRK-52E cells exposed to LPS, and these increases were significantly inhibited by sRAGE

treatment (Fig. 9).

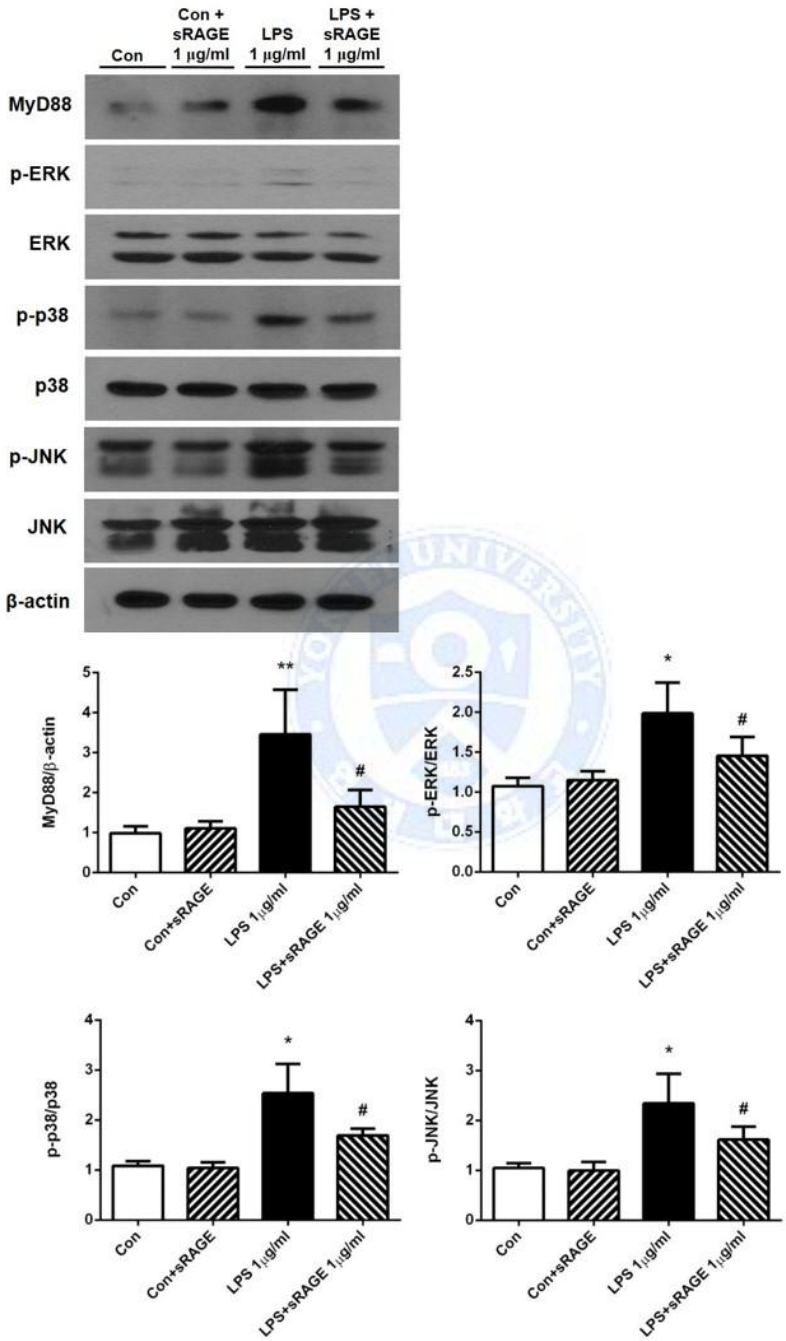

Figure 9. A representative western blot of MyD88, phospho-ERK (p-ERK),

phospho-p38 (p-p38), and phospho-JNK (p-JNK) in control (Con), Con+sRAGE (1 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), and LPS+sRAGE groups (Representative of six blots). Compared to control cells, MyD88, p-ERK, p-p38 and p-JNK protein expression were significantly increased in NRK-52E cells exposed to LPS, these increase were significantly inhibited by sRAGE treatment. $*P < 0.05$ and $**P < 0.01$ vs. Con group, $^{\#}P < 0.05$ vs. LPS group.

10. sRAGE abrogates LPS-induced nuclear translocation of NF-κB in NRK-52E cells

To explore the impact of sRAGE on LPS-induced NF-κB activation, nuclear translocation of NF-κB was determined by investigating the changes in NF-κB protein expression in both cytosolic and nuclear fractions. In addition, ICAM-1 protein expression as a mediator of inflammatory cascades was examined. NF-κB protein expression in the cytosolic fraction was significantly decreased at 12hr after the administration of 1 μg/ml LPS ($P < 0.05$), and this decrease was significantly ameliorated by sRAGE treatment ($P < 0.05$). In contrast, NF-κB expression in the nuclear fraction was significantly increased by LPS ($P < 0.05$), and sRAGE treatment significantly attenuated this increase ($P < 0.05$). ICAM-1 protein expression was also upregulated in LPS-stimulated NRK-52E cells, and this increase was significantly inhibited by sRAGE (Fig. 10).

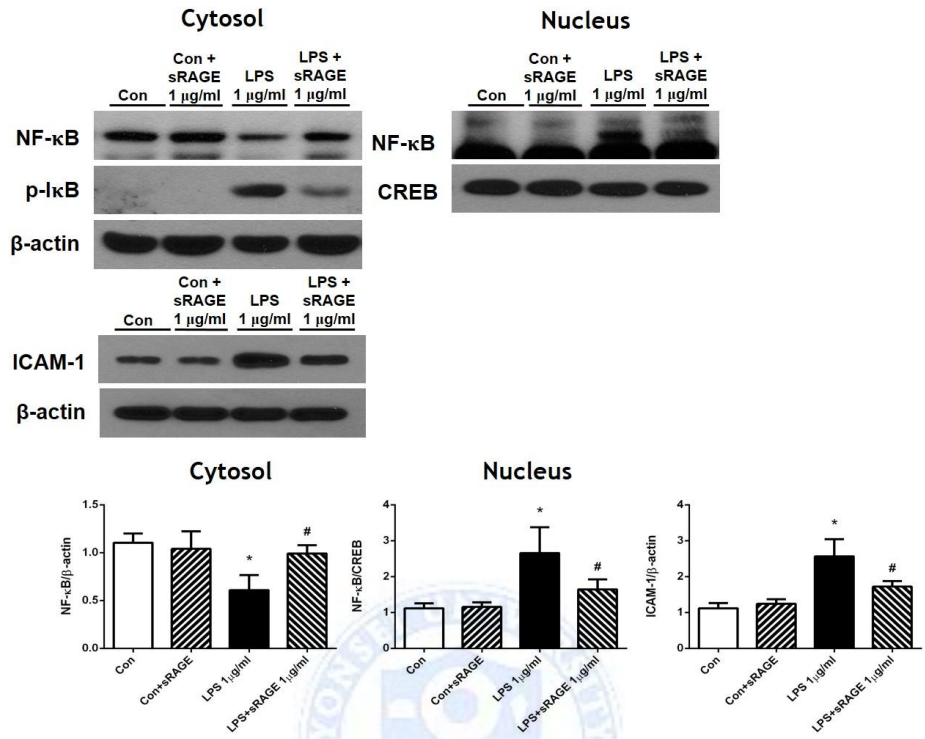

Figure 10. A representative western blot of NF-κB protein expression in the cytosolic and nuclear fractions in control (Con), Con+sRAGE (1 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), and LPS+sRAGE groups (Representative of six blots). Compared to Con NRK-52E cells, NF-κB protein expression in the cytosolic fraction was significantly decreased at 12 hr after the administration of LPS, and this decrease was significantly ameliorated by sRAGE treatment. In contrast, NF-κB protein expression in the nuclear fraction was significantly increased by LPS, and sRAGE treatment significantly attenuated this increase. ICAM-1 protein expression showed a similar pattern. $^{*}P < 0.05$ vs. Con group, $^{\#}P < 0.05$ vs. LPS group.

11. sRAGE abrogates LPS-induced apoptosis in NRK-52E cells

To clarify whether LPS induces apoptosis and sRAGE protects LPS-induced apoptosis in NRK-52E cells, the protein expression of apoptosis-related

molecules were determined. Hoechst 33342 staining was also performed. Compared to control NRK-52E cell, Bax and cleaved caspase-3 protein expression was significantly increased, while Bcl-2 protein expression was significantly decreased in LPS-stimulated cells, and these changes were significantly ameliorated by sRAGE treatment (Fig. 11).

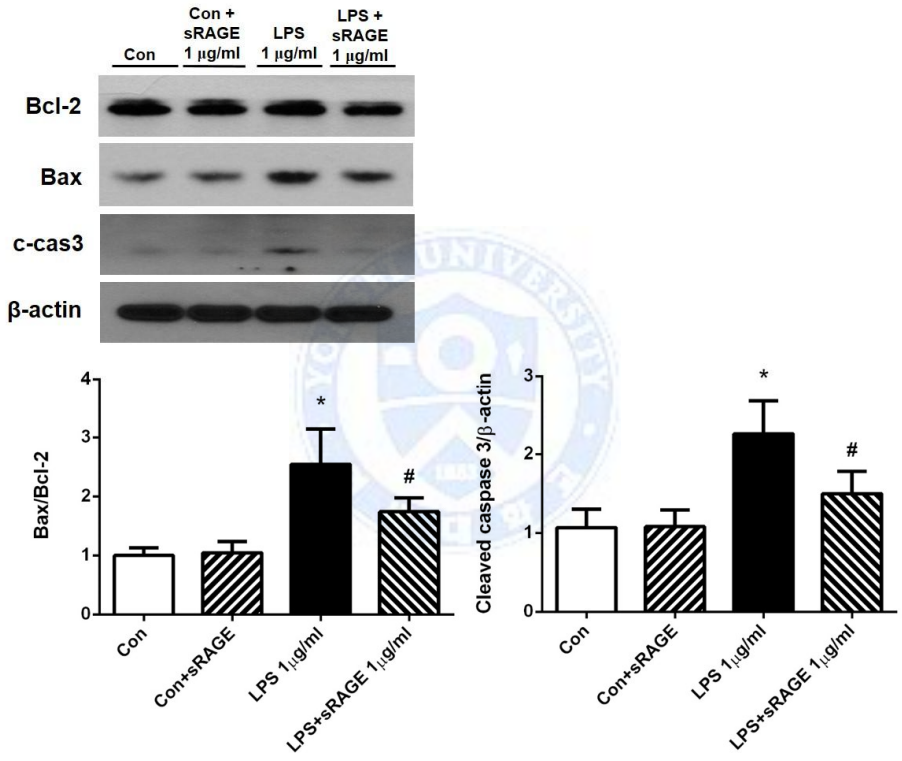

Figure 11. A representative western blot of Bax, cleaved caspase-3 (c-cas3), and Bcl-2 protein expression in control (Con), Con+sRAGE (1 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), and LPS+sRAGE groups (Representative of fix blots). Compared to control NRK-52E cells, Bax and c-cas3 protein expression were significantly increased, while Bcl-2 protein expression was significantly decreased in LPS-stimulated cell, and these changes were significantly ameliorated by sRAGE

treatment. *$P < 0.05$ vs. Con group, $^{\#}P < 0.05$ vs. LPS group.

Apoptotic cells assessed by Hoechst 33342 staining were also significantly increased in LPS-stimulated NRK-52E cells, and sRAGE significantly attenuated this increment in apoptotic cells induced by LPS (Fig. 12).

Finally, the protective effect of RAGE inhibition on LPS-induced inflammation and apoptosis was verified by using 100 nM RAGE siRNA. The increases in ICAM-1 and cleaved caspase-3 protein expression in LPS-stimulated cells were significantly abrogated by RAGE siRNA (Fig. 13).

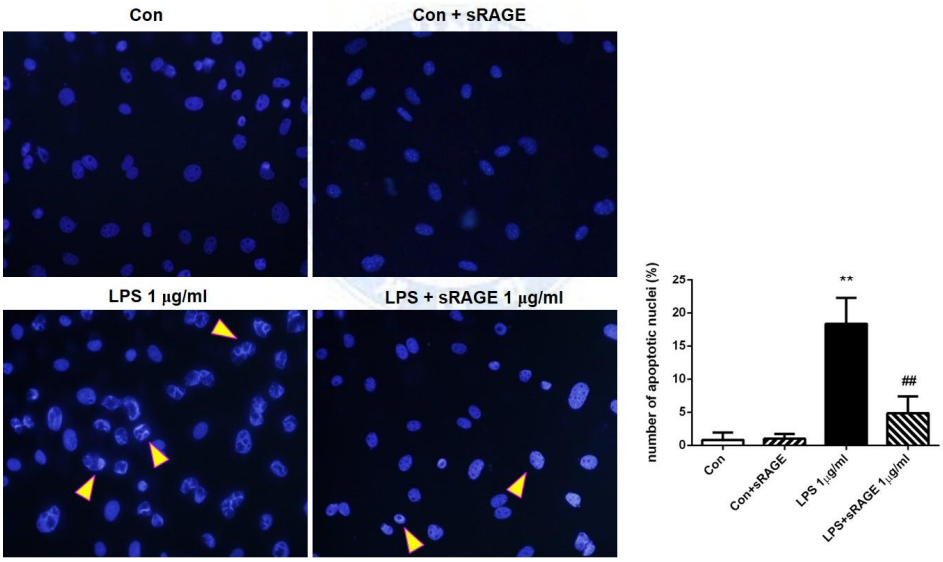

Figure 12. Apoptotic cells assessed by Hoechst 33342 staining in control (Con), Con+sRAGE (1 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), and LPS+sRAGE groups. Apoptotic cells (arrowheads) were significantly increased in LPS-stimulated NRK-52E cells, and sRAGE significantly attenuated this increment in apoptotic cells induced by LPS. **$P < 0.01$ vs. Con group, $^{\#\#}P < 0.01$ vs. LPS group.

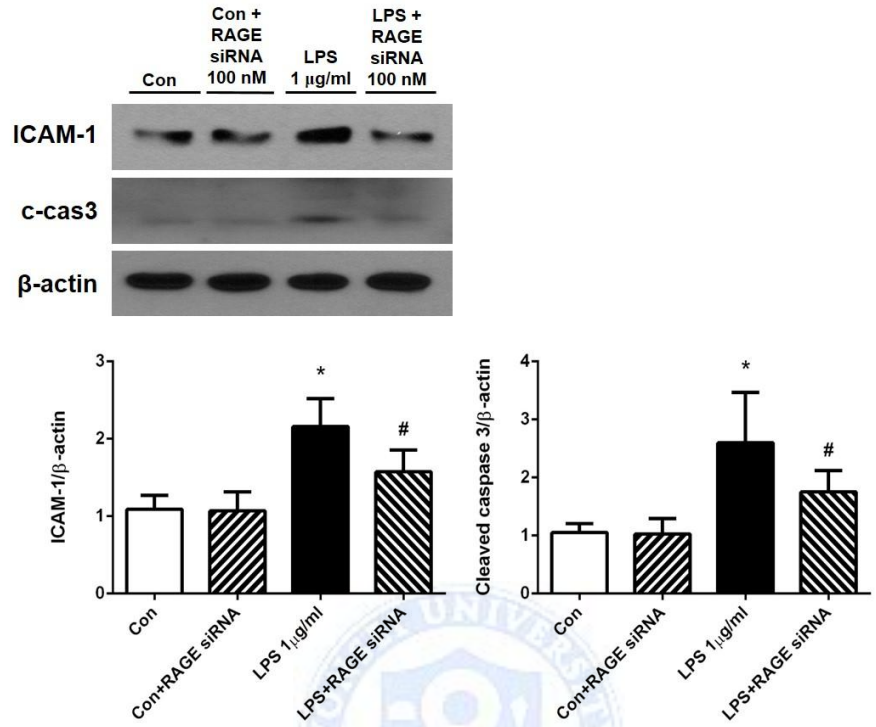

Figure 13. A representative western blot of ICAM-1 and cleaved caspase-3 (c-cas3) protein expression in control (Con), Con+sRAGE siRNA (100 nM), lipopolysaccharide (LPS, 1 μg/ml), and LPS+RAGE siRNA groups (Representative of six blots). The increase in ICAM-1 and c-cas3 protein expression in LPS-stimulated cells were significantly abrogated by RAGE siRNA. $^{*}P < 0.05$ vs. Con group, $^{\#}P < 0.05$ vs. LPS group.

V. DISCUSSION

The results of the present study showed that EN-RAGE and sRAGE levels were significantly increased and that high sRAGE levels were associated with better survival rates in severe septic AKI patients. In addition, it was demonstrated that sRAGE treatment improved not only renal function but also systemic inflammation in CLP-induced septic AKI animal models. Moreover, administration of sRAGE inhibited NF-κB-mediated tubulointerstitial inflammation and tubular cell apoptosis in septic AKI both *in vivo* and *in vitro*. These data suggests that RAGE modulation by sRAGE may serve as a potential therapeutic target for AKI in severe sepsis patients.

RAGE has emerged as a central regulator for systemic inflammation and subsequent tissue damages.[31] Binding of RAGE with various kinds of ligands such as HMGB1 as well as AGEs results in pro-inflammatory gene activation and consequent propagation of inflammatory cascade,[32] and thus the interactions between RAGE and its ligands are considered to be associated with a range of inflammatory diseases including sepsis. Meanwhile, the extracellular domain of RAGE is cleaved to sRAGE, which is secreted into systemic circulation and exerts protective effects against RAGE-induced inflammatory tissue damages.[22] Previous studies showed that RAGE was accumulated and existed in several variants in patients with reduced renal function.[33, 34] Serum levels of sRAGE and EN-RAGE were also increased in patients with renal insufficiency including ESRD patients.[21, 35] Furthermore, serum sRAGE concentrations was negatively associated with inflammation and oxidative

stress in chronic kidney disease (CKD) patients.[21, 33] In this study, I demonstrated that sRAGE levels was significantly increased in septic AKI patients compared to control and even to dialysis subjects. In patients with septic AKI, in addition, increased serum concentrations of sRAGE had a protective impact on survival in these patients. Based on these findings, it was inferred that sRAGE might be increased by a counter-regulatory mechanism against enhanced RAGE expression to protect from oxidative stress, inflammation, and pro-apoptotic injury in severe septic AKI patients.

In the past, sepsis-induced renal injury was considered consequences of tubular ischemia and subsequent acute tubular necrosis due to hemodynamic instability, but recent robust studies found that non-hemodynamic injury including inflammation and coagulation abnormality played a critical role in the development of renal tubular cell damage in sepsis.[36] Circulating pro- and anti-inflammatory mediators induce the recruitment of inflammatory cells and tubular cell apoptosis. Among them, HMGB1, which is released from injured cells and macrophages, has been suggested as one of late-appearing pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs), and regulates microbial-induced inflammation and LPS-induced cellular and tissue injuries.[37-39] Moreover, it is an important ligand of RAGE, which is activated and triggers inflammatory processes in sepsis and acute inflammation. A previous study also showed that HMGB1-neutralizing antibody reversed sepsis in mice.[40] Taken together, it is inferred that HMGB1 may play an important role in the pathogenesis of sepsis. The current study demonstrated that HMGB1 and RAGE protein expression were significantly increased in the

kidney of sepsis mice and LPS-treated tubular cells and that sRAGE ameliorated CLP- and LPS-induced tubulointerstitial inflammation and tubular cell apoptosis both *in vivo* and *in vitro*. Interestingly, the increases in RAGE and HMGB1 expression in tubular cells under septic conditions were also significantly attenuated by sRAGE treatment. If RAGE expression was further induced by continuous ligand activation and HMGB1 was shed from injured cells,[38, 41] RAGE and HMGB1 expression could be influenced by sRAGE via mitigating RAGE-mediated induction of various chemokines and adhesion molecules and release of HMGB1 associated with tubular cell apoptosis. In accordance with the present study, Lee et al. also found that sRAGE abrogated angiotensin-II induced RAGE expression in the aorta of apolipoprotein E deficient mice.[27]

HMGB1 exerts pro-inflammatory effects by binding primarily to RAGE and/or IL-1 receptor (IL-1R).[42] Recently, it has been revealed that HMGB1 also interacts with TLR2/4.[43] On the other hand, LPS, a well-known ligand of TLRs, is an endotoxin induced by exogenous pathogens in sepsis.[44] Based on the results of previous studies showing that interaction of HMGB1 and LPS with RAGE and TLR led to signal transduction propagation via MyD88 and the MAP kinase pathway and cytokine production,[45] it was suggested that blocking RAGE signaling could ameliorate HMGB1 and LPS-induced inflammatory response in sepsis.[13] In this study, I demonstrated that MyD88 and MAP kinase including ERK, p38, and JNK were activated in the kidney of CLP mice and LPS-treated tubular cells, and sRAGE treatment significantly attenuated these changes in renal tubular cells under septic conditions.

Importantly, signaling through RAGE leads to NF-κB activation by nuclear translocation of NF-κB and in turn induces the transcription of inflammation-associated genes such as ICAM-1, which is a cell surface glycoprotein and plays a major role in the infiltration process of macrophages and monocytes.[46] Once the macrophages/monocytes are infiltrated and activated, they release lysosomal enzymes, nitric oxide, reactive oxygen species, tumor necrosis factor-α, IL-1, and transforming growth factor-β, and consequently promote renal injury including tubular cell apoptosis.[47, 48] The results of the current study found, that NF-κB protein expression and nuclear translocation of NF-κB were significantly increased in the kidney of CLP mice and LPS-treated NRK-52E cells along with, an increase in ICAM-1 expression. Furthermore, the number of infiltrated macrophages with the renal tubulointerstitium was significantly higher in CLP mice. In addition, administration of sRAGE significantly abrogated the increases in nuclear translocation of NF-κB, ICAM-1 expression, and macrophage infiltration in the kidney of CLP mice. These findings surmised, that sRAGE ameliorated renal tubular injury via its anti-inflammatory effect in sepsis animal models. *In vitro*, however, tubular cell apoptosis induced by LPS was also significantly attenuated by RAGE inhibition. Taken together, the impact of sRGAE on renal tubular injury seems to be mediated by not only mitigating macrophages/monocytes infiltration but also its direct beneficial effects on renal tubular cells independently of inflammation.

Various immuno-competent cells have been shown to play a critical role in kidney injury and repair. Among them, Treg cells were demonstrated to be involved in immunomodulation in AKI.[49, 50] A recent study found that the

number of Treg cells was increased in heat preconditioned splenocytes and contributed to the renoprotective effect of heat preconditioning.[49] Tatura et al. also showed that $Foxp3^+$ Treg cells were increased in murine sepsis model and depletion of Treg cells aggravated the severity of sepsis in these mice.[51] In contrast, another study demonstrated that splenectomy reduced circulating HMGB1 levels in CKD-sepsis animal model.[52] The results of the present study revealed that the number of $CD4^+CD25^+Foxp3^+$ cells in the spleen were increased and this increase was further augmented by sRAGE treatment in CLP mice, suggesting that expanded Treg cells by sRAGE may partly contributed to the improvement in sepsis-induced AKI via inhibiting RAGE signaling. Collectively, sRAGE may also exert a protective impact against septic AKI through expansion of immune modulatory cells including Treg cells in addition to its anti-inflammatory and direct anti-apoptotic effect on renal tubular cells.

V. CONCLUSION

To clarify whether sRAGE has a renoprotective effect on S-AKI, I have examined the changes in tubulointerstitial inflammation, tubular cell apoptosis, and various intracellular signal transduction pathway by sRAGE under septic conditions both *in vivo* and *in vitro*.

1. Compared to control mice, HMGB1 and RAGE protein expression were significantly increased in the kidney of CLP mice, these increases were significantly abrogated by sRAGE treatment.

2. BUN, and serum creatinine and IL-6 levels were significantly higher in CLP mice compared to control, and administration of sRAGE significantly ameliorated these increases.

3. MyD88, p-ERK, p-p38, and p-JNK protein expression were significantly increased in the kidney of CLP mice, and these increases were significantly attenuated by sRAGE treatment.

4. Renal NF-κB phosphorylation and ICAM-1 protein expression were significantly increased in CLP mice, and sRAGE significantly abrogated the increases in phospho-NF-κB and ICAM-1 protein expression in these mice.

5. Cleaved PARP and cleaved caspase-3 protein expression were significantly increased in the kidney of CLP mice, and these increases were significantly ameliorated by sRAGE.

6. The number of apoptotic cells and infiltrated macrophages were significantly higher in the kidney of CLP mice, and administration of s RAGE significantly attenuated these increases in CLP mice.

7. The number of $CD4^{+}CD25^{+}Foxp3^{+}$Treg cells, assessed by FACS analysis,

was increased in the spleen of CLP mice, and this increase was further augmented by sRAGE treatment.

8. HMGB1 and RAGE protein expression were significantly increased in LPS-stimulated NRK-52E cells, and sRAGE significantly abrogated these in these cells.

9. MyD88, p-ERK, p-p38, and p-JNK protein expression were significantly increased in NRK-52E cells exposed to LPS, and these increases were significantly ameliorated by sRAGE.

10. NF-κB protein expression was significantly decreased in the cytosolic fraction, while its expression was significantly increased in the nuclear fraction by LPS, and these changes were significantly attenuated by sRAGE treatment. ICAM-1 protein expression showed a similar pattern.

11. Bax and cleaved caspase-3 protein expression were significantly increased, whereas Bcl-2 protein expression was significantly decreased in LPS-stimulated NRK-52E cell, and sRAGE significantly abrogated these changes.

12. The number of apoptotic cells, assessed by Hoechst 33342 staining, was also significantly higher in NRK-52E cells exposed to LPS, and this increment was significantly ameliorated by sRAGE.

13. The increase in ICAM-1 and cleaved caspase-3 protein expression in LPS-stimulated NRK-52E cells were also significantly attenuated by the administration of RAGE siRNA.

In conclusion, I demonstrated that sRAGE might play renoprotective role in S-AKI through various mechanisms; an anti-inflammatory effect, direct anti-apoptotic impact on renal tubular cells, and expanding immune modulatory cells

including regulatory T cells. These data suggests that RAGE modulation by sRAGE may serve as a potential therapeutic target for AKI in severe sepsis patients.

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ABSTRACT (IN KOREAN)

패혈성 급성 신손상 모델에서 soluble receptor for advanced glycation end-products (sRAGE)의 신보호 효과

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패혈증에 의한 급성 신손상을 동반한 환자의 이환율 및 사망률은 매우 높기 때문에 임상적으로 매우 심각할 뿐만 아니라 향후 만성 신질환으로의 이환을 포함한 각종 합병증 발생의 중요한 원인으로 여겨지고 있다. 최근의 연구 결과들에 의하면 이러한 패혈성 급성 신손상 발생의 중요한 기전으로 여러 가지 염증 매개 물질의 과잉이 직접적인 연관이 있으며, 그 중에서도 advanced glycosylation end-product receptor (RAGE)의 활성화가 이러한 패혈성 급성 신손상의 염증 반응과 밀접한 관련이 있는 것으로 밝혀졌다. 본 연구에서는

패혈성 급성 신손상에서 RAGE의 역할과 soluble RAGE(sRAGE)의 효과 및 신보호 기전을 규명하여 향후 급성 신손상을 동반한 환자에서 sRAGE의 임상 적용 가능성을 알아보고자 하였다. CLP (cecal ligation and puncture)로 유발된 패혈성 신손상 동물 모델과 lipopolysaccharide (LPS)로 자극한 신세뇨관 세포 (NRK-52E 세포)를 이용하여 실험을 진행하였다. CLP군의 신장 내 HMGB1과 RAGE의 발현은 의미있게 증가되었으며, 이러한 증가는 sRAGE 전처리로 의의있게 억제되었다. 혈청 BUN, Creatinine, 그리고 IL-6 농도도 CLP군에 비하여 sRAGE 투여 군에서 유의하게 감소되었다. 또한, CLP 마우스의 신장에서 증가되었던 PARP, cleaved caspase-3, 그리고 NK-κB의 활성과 염증세포의 침윤 및 세포사멸이 sRAGE 전처치로 의미있게 감소되었다. sRAGE는 LPS로 자극한 신세뇨관 세포를 대상으로 한 실험에서도 유사한 효과를 나타내었다. 이상의 결과로, sRAGE가 패혈성 급성 신손상에서 RAGE를 억제함으로써 RAGE에 의하여 매개되는 염증 반응 물질에 의한 염증 반응 및 세포 사멸을 억제함을 알 수 있었으며, 향후 급성 신손상을 동반한 패혈증 환자의 치료에도 활용될 수 있을 것으로 생각된다.

핵심 되는 말: RAGE, sRAGE, 패혈증, 급성 신손상